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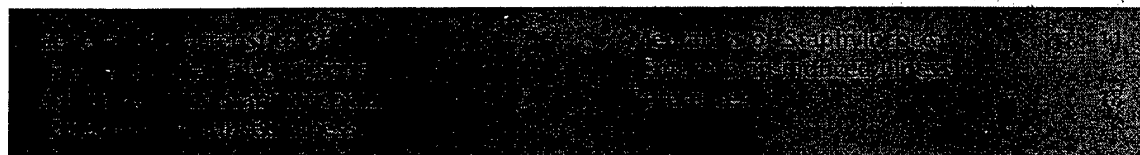
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The Staphylococcal Enterotoxins

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General Characteristics of Staphylococcal Enterotoxins

The staphylococcal enterotoxins (SEs) are a family of related proteins whose biological toxicities include staphylococcal food poisoning (SFP), various skin disorders, toxic shock syndrome (TSS), and possible involvement in auto-immune disorders. The SEs, named sequentially by letter, include SEA, B, C1, C2, C3, D, E, G, H, I, J and toxic shock syndrome toxin 1 (TSST-1). They have been characterised as superantigens, because of their massive impact on the host immune system, and their interaction with lymphoid cells has been widely studied. SE also exert effects on endothelial cells, kidney proximal tubule cells, synovial cells and human platelets. A large number of studies in the literature have examined. The genetic, biochemical and biological properties of staphylococcal enterotoxins have been extensively reviewed (Bergdoll, 1972; Holmberg and Blake, 1984; Bergdoll *et al.*, 1985; Marrack and Kappler, 1990; Leung *et al.*, 1993a; Bohach, 1997; Jablonsky *et al.*, 1997; Dinges *et al.*, 2000).

One or more of these exoproteins are produced and secreted by certain isolates of the Gram-positive bacterium *Staphylococcus aureus*. They are members of a larger group of proteins produced by *Staph. aureus* and *Streptococcus pyogenes*, termed pyrogenic

exotoxins, which share the property of inducing fever and shock in human and non-human primates. Only SEs, however, are associated with staphylococcal food poisoning.

Staphylococcal Food Poisoning

Staphylococcal food poisoning (SFP) is a form of enteritis resulting from ingestion of food contaminated with preformed staphylococcal enterotoxin, and thus SFP is an intoxication rather than a disease of infection (Bergdoll *et al.*, 1974). Contamination with toxin results from conditions, such as poor refrigeration or undercooking, that promote growth of toxin-producing *Staph. aureus*. After toxin ingestion, there is rapid (1-4 hours) onset of vomiting that may or may not be accompanied by nausea and diarrhoea. Vomiting in the absence of high fever is characteristic of SFP and this usually resolves in 24-48 hours. Even at low ingestion doses, in experimental studies in non-human primates and cases of people accidentally exposed, symptoms include dizziness, abdominal colic, possible vasoconstriction in extremities resulting in extreme muscular weakness with icy-cold feet and hands and concomitant vasodilatation in the kidneys and other organs (Pereira *et al.*, 1994; Mattix *et al.*, 1995; Carmo *et al.*, 2001). Anorexia may last up to 7 days after even a mild exposure. Dehydration is one of the

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2 GASTRO-INTESTINAL INFECTIONS: TOXIN-ASSOCIATED DISEASES

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most common complications of SFP, and the illness may lead to lethal shock. The groups most affected are young children and older adults with other persistent health problems (Weed *et al.*, 1943; Pereira *et al.*, 1994; Carmo *et al.*, 2000).

Contaminated Food Products

There is a long history of staphylococcal enterotoxins causing serious illnesses from accidentally contaminated food products. A well-documented example is induction of lethal shock in infants and small children after drinking SEB-contaminated goat's milk (Weed *et al.*, 1943). Dairy products are among the foods frequently contaminated with toxin or toxin-producing staphylococci. The two most frequently identified routes of contamination are mastitis of cows (Garcia *et al.*, 1980) and goats (Bezek and Hull, 1995), and human carriers involved in milking (Adesiyun *et al.*, 1998). *Staph. aureus* has been shown to produce enterotoxins A, B, C and/or D in the milk of sheep (Bautista *et al.*, 1988), goats (Valle *et al.*, 1990; Adesiyun, 1994, 1995; Adesiyun *et al.*, 1998) and cattle (Tatini *et al.*, 1971a; Olsvik *et al.*, 1982; Abo-Elnaga *et al.*, 1985; Kayihura *et al.*, 1987; Umoh *et al.*, 1990). Dairy products may also contain toxins and/or toxin-producing *Staph. aureus* (Rose *et al.*, 1989; Gilmour and Harvey, 1990; Burdova *et al.*, 1994) and contaminated cheese, frequently made from raw milk, may harbour enterotoxins or bacteria or both (Tatini *et al.*, 1971b, 1973; Ibrahim *et al.*, 1980; Rosec *et al.*, 1997). SEA or toxin-producing *Staph. aureus* have been identified in cream-filled cakes, cheese and breast milk in Brazil (Pereira *et al.*, 1994, 1995), in a 'black pudding' commonly served in Trinidad (Adesiyun and Balbirsingh, 1996), beef from abattoirs (Desmarchelier *et al.*, 1999); the most heavily contaminated were the chicken carcasses (Ombui *et al.*, 1992).

Asymptomatic Human Carriers

The common source of bacterial contamination of food are food handlers who are asymptomatic carriers of SE-producing *Staph. aureus*. In one study almost half of nasal staphylococci of hospital food handlers produced high levels of enterotoxins (A > B, C >> D, E), and some produced several toxins (Reali, 1982). A study of 300 food handlers in Spain showed that 12% were carriers of enterotoxigenic staphylococci (Francisco Polledo *et al.*, 1985). In Kuwait City, 27% of 500 restaurant workers were nasal carriers of *Staph. aureus* and nearly all isolates produced staphylococcal enterotoxins, which is not higher than in the general population (Al Bustan *et al.*, 1996). After a food poisoning outbreak in a restaurant in Brazil,

enterotoxigenic staphylococci were found in the noses, throats and under the fingernails of food handlers (Carmo *et al.*, 2000). In a study of 821 healthy persons, 31% were carriers of enterotoxigenic staphylococci (57% of food handlers, 14% of students) and half produced SEA, and the others produced SEB and C. Isolates from 40% of persons receiving medical treatment may produce SEs (Sourek *et al.*, 1979). TSST-1 is produced by nasal isolates (Olusanya and Naidu, 1991).

Enterotoxigenic staphylococci have been isolated from about 30% of the adult population and these bacteria are the obvious source of many of the extensive food poisoning outbreaks. It has been shown that the growth of bacteria inoculated is minimal if the food is rapidly and thoroughly refrigerated (Anunciacao *et al.*, 1995). In one incident a very large cake was inadequately cooled before inserting the cream filling and those eating it became violently ill, and SEA was detected in the remaining cream filling (Pereira *et al.*, 1994).

Antitoxin Profiles of Human Carriers

It is not known whether the staphylococci of human carriers produce the toxins at sites of colonisation. To address this question, antibody levels have been examined in these individuals, on the assumption that carrier status may be reflected in higher antitoxin titres. Serum antibody levels were compared in patients with staphylococcal infections and healthy carriers. An almost identical proportion of the staphylococci from each group (93 versus 94%) produced enterotoxins and the antibody patterns to SEA, B, C, D, E and TSST-1 were virtually identical (Solino Noleto *et al.*, 1986). Similarly, of laboratory personnel working with SEB, 85% had antibodies as compared with 23% in the general population (Jozefczyk, 1973). This may reflect an age-related variation in antibody titre, since in another study a lower percentage of students, as compared with the general population, were carriers of enterotoxigenic staphylococci (Sourek, 1980). Sera from patients with TSS had lower levels of antibodies to staphylococcal enterotoxins A, B and C than controls, and a combinations of TSST-1 and SEC was the most lethal (Crass and Bergdoll, 1986).

In view of the association of superantigens with rheumatoid arthritis, Tabarya and Hoffman (1996) compared rheumatoid arthritis patients and controls, including the healthy spouses of patients, for nasal carriage of *Staph. aureus* and antibody titre to TSST-1 and SEA, as a measure of past exposure to the toxins. The patients and controls had a carrier rate of 50% and 35%, respectively, and bacteriophage typing of the isolates suggested a marked differences between strains

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isolated from the two groups. The rheumatoid arthritis patients had significant higher IgG and IgA antibody levels to TSST-1 than the controls, unrelated to rheumatoid factor titres or HLA-DR type.

Food-borne illnesses due to SE range in severity and outcome depending on the dose of the toxin consumed and general health status. Attempts have been made to determine the age-related severity of SE exposure, but definitive answers are not available. Questions remain about the involvement of the superantigenic properties of SEs in the emetic response. These are based on observations that genetically altered (Harris *et al.*, 1993a; Hovde *et al.*, 1994) and chemically altered (Scheuber *et al.*, 1987a, b) SEs retain T-cell mitogenic activity but lose emetic and functional activities. Studies with SE fragments (Bohach, 1997) and specific antisera (Alber *et al.*, 1990) support the notion that the emetic action of SE is separate from its superantigenicity. The cells and receptors in the gut to which the SEs bind have not been clearly defined. Mast cell interactions, neuro-peptide generation and inflammatory mediators appear to play a role (Scheuber *et al.*, 1987a; Jett *et al.*, 1990; Boyle *et al.*, 1994). A study in mice, however, showed that SEB, administered intragastrically, induced mucosal T-cell expansion and up-regulated cytokine mRNA (Spiekermann and Nagler-Anderson, 1998). SEB produces severe villus atrophy and crypt cell hyperplasia in human fetal gut explant cultures (Pender *et al.*, 1998).

Toxic Shock Syndrome

TSS is a potentially lethal disease defined by high fever, severe hypotension, oedema, diffuse erythematous rash, desquamation of skin and dysfunction of three or more organ systems, frequently including kidneys and/or lungs (Dinges *et al.*, 2000). This toxin-mediated systemic disease was first observed in non-systemic infections by SE-producing *Staph. aureus* (Todd *et al.*, 1984). Subsequently, another *Staph. aureus* toxin, designated as toxic shock syndrome toxin 1 (TSST-1), was shown to be associated with TSS in menstruating women and in non-menstrual cases (Dinges *et al.*, 2000). Pyrogenic exotoxins of *Strep. pyogenes* may also cause TSS (Bohach *et al.*, 1990). These toxins share structural and biological properties with SEs, such as enhancement of endotoxic shock, but only SEs induce vomiting.

SE-induced illness, unrelated to contaminated food, and leading to lethal shock has been recorded in the literature. Scald burn victims had been described, who had diarrhoea, vomiting, general malaise, pyrexia, tachycardia and tachypnoea within 24–48 hours of a

burn injury. Sequestration of peripheral blood lymphoid cells and low haemoglobin concentrations were evident 3–4 days after the burn, and about half of the children died of an illness typical of lethal shock (Farmer *et al.*, 1985; Heywood and al-Essa, 1990; McAllister *et al.*, 1993; Marodi *et al.*, 1995). In another study about half of young children with burn injuries had antibodies to TSST-1 but this did not alter the outcome (Childs *et al.*, 1999).

There are numerous different cases, all with similar patterns of illness, in which SEA or SEB induced TSS. Some examples are an influenza-like illness in which isolates produced SEB or TSST-1 (MacDonald *et al.*, 1987). Other studies show that for menstrual TSS, SEA was frequently found along with TSST-1 (Kain *et al.*, 1993) while for non-menstrual TSS, SEB was the predominant toxin and TSST-1 was not present (Lee *et al.*, 1992). Interestingly, the non-menstrual TSS patients had a higher incidence of previous antimicrobial treatment (46% versus 16%). The course of symptoms differed in the two groups, with non-menstrual TSS showing more frequent renal and CNS complications (Kain *et al.*, 1993).

Bacteraemia due to methicillin-resistant *Staph. aureus* (MRSA) is most severe for the various enterotoxin types produced, with a mortality rate significantly higher in patients over 51 years of age than in the younger patients (50% versus 4%) (Nada *et al.*, 1996).

Genetic Characteristics

Staphylococcal enterotoxins share many structural and functional similarities, but each also has distinct features. They are relatively heat-resistant and resistant to protease digestion by gastric enzymes. In early studies five antigenically distinguishable SE serotypes, SEA, SEB, SEC, SED, SEE, were identified. SEC consists of three additional subtypes, SEC1, 2 and 3. Four additional types (SEG, SEH, SEI and SEJ) have been identified by use of recombinant DNA methods (Ren *et al.*, 1994; Munson *et al.*, 1998; Zhang *et al.*, 1998). Two new types of SEC, SEC-ovine and SEC-bovine, have also been found (Marr *et al.*, 1993). All of the SEs tested (SEA–SEI) can induce vomiting, ~~only a few of the toxin serotypes are frequently associated with food poisoning outbreaks, but this may relate to availability of reagents. Toxin serotypes frequently associated with food poisoning outbreaks are SEA, SED, SEC and SEB, with SEA the predominant type (Holmberg and Blake 1984). In contrast, SE serotypes associated with non-menstrual toxic shock are primarily SEB and SEC (Bohach *et al.*, 1990).~~

The regulation of SE expression in culture and in the location of their genes differ. SEB and SEC are

for detection of the toxins.

infection

structures
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the second domain and one of the α -helices in the groove between the two domains.

The sequence diversity between the SEs is reflected in the differences each displays in its interaction with MHC II and T-cell receptors. A schematic representation of this interaction is shown in Fig. 2. The SEs vary in affinity for different MHC II types and for binding sites on MHC II (Herrmann *et al.*, 1989). Competitive binding indicates that the SEB/C/G family and SEA have a common binding site on MHC II (Thibodeau *et al.*, 1994), but that SEA primarily binds at additional site that requires zinc ions as a co-factor (Fraser *et al.*, 1992). This dual binding ability for SEA may add to its potency. SEE and SED have a similar zinc requirement (Svensson *et al.*, 1997). In addition, each SE has a distinct repertoire of V β T-cell receptor subunits with which they react (Choi *et al.*, 1990).

Chemical modification of SE proteins to define the structural features involved in their function suggested that certain amino acid residues were important for function or structural folding of the molecule (Stelma and Bergdoll, 1982). Enzymatic cleavage of the toxin peptide (Spero *et al.*, 1975) and deletion mutagenesis (Harris *et al.*, 1993b) of the toxin gene indicated that amino acid sequences at both the N-terminal and C-terminal regions of the molecule were needed for function. Similarly, studies with peptide fragments showed that multiple regions were involved in mitogenesis (Jett *et al.*, 1994; Hoffman *et al.*, 1996; Johnson *et al.*, 1996).

Site-specific mutagenesis of several SEs and X-ray crystallographic analysis of SEB bound to MHC II and

SEB and SEC bound to T-cell receptor α chain have added greater detail to the features that affect binding of SEs to these molecules (Kappler *et al.*, 1992; Jardetzky *et al.*, 1994; Fields *et al.*, 1996; Leder *et al.*, 1998; Li *et al.*, 1998a b). Multiple conserved residues, mainly in the smaller domain structure of the toxin, but also in the N- and C-terminal regions, contribute to the binding of SEB to the β subunit of MHC II. This binding occurs outside of the peptide antigen-binding groove. A small cleft formed in the upper area between the two structural domains of SEB and SEC contains multiple non-conserved residues from three different regions of the toxin sequence which mediate binding to specific loop regions of the V β chain of the T-cell receptor. These toxin regions bind to the backbone portion of the V β protein chain, rather than to the residues. The positioning of this backbone area is different in different V β types and the residues in the TCR-combining site in each SE are also different, accounting for the variability in affinity of each SE to particular V β types.

Studies with peptide fragments suggest that another region in a β strand of the larger toxin structural domain, not recognised by crystallographic or mutational analysis, may be involved in receptor binding in addition to those described above (Jett *et al.*, 1994; Hoffman *et al.*, 1996; Johnson *et al.*, 1996; Di Stefano *et al.*, 1998). No significant conformational changes in either toxin or receptor has been observed in toxin-receptor complexes, suggesting that the assembly of multi-protein complexes on the cell surface, rather than receptor conformational changes, may initiate signal transduction events within the cell (Li *et al.*, 1998).

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both $\alpha + \beta$

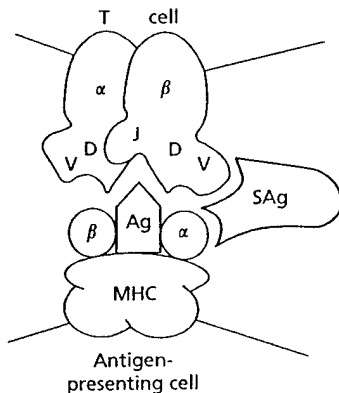


Fig. 2 Schematic representation of superantigen (SAg) interaction with the MHC of the antigen-presenting cell, coupled with the V β subunit on the T cell. Reprinted from Leung DYM, Huber BT, Schlievert PM (eds) (1997) *Superantigens: Molecular Biology, Immunology, and Relevance to Human Diseases*, p. 134, with permission from Marcel Dekker.

Cell Targets for Staphylococcal Enterotoxin-Induced Illness

Superantigenic Characteristics of Staphylococcal Enterotoxins

T-cell Antigen Response

T lymphocytes constitute the lineage of lymphoid cells that is primarily responsible for mediating cellular immunity. They are derived from haematopoietic precursors and undergo a complex affinity selection-driven maturation process in the thymus, during which cells are selected for survival that recognise foreign antigenic peptides presented in the context of self class II major histocompatibility complex molecules (MHC).

Classical α/β receptor T cells are grouped into two functional sets: (1) cytotoxic T lymphocytes (CTL), which are primarily responsible for induction of

apoptosis in cells infected with intracellular parasites (mainly viruses); and (2) helper T lymphocytes, which are further divided into two subsets, designated TH1 and TH2, based on their pattern of pro- or anti-inflammatory lymphokine secretion. The primary function of helper T cells is to recognise extracellular antigens, presented in the context of the class II MHC molecule on the surface of the antigen-presenting cell (APC), and to deliver the signal to the B cell. The B cell, in turn, is responsible for mounting the antibody response (Oppenheim *et al.*, 1990).

Structure of T-cell Receptor

Most T cells express the classical α/β T-cell antigen receptor (TCR), the principal participant in T-cell antigen recognition. The disulphide-linked α/β heterodimer, directly responsible for the recognition and binding of the antigen, is closely associated with six additional polypeptide chains, γ , δ , two ϵ , and two ζ , collectively referred to as the CD3 complex (Paul *et al.*, 1993). The gene encoding the α/β heterodimer belongs to the immunoglobulin superfamily and is subject to gene rearrangement processes similar to those typical of immunoglobulin molecules, with the exception of the process of somatic mutation which is unique to immunoglobulin molecules. As a result, the α/β dimer exhibits a high degree of polymorphism, but not to the same ^{extent} degree as immunoglobulins. By contrast, the CD3 complex is non-polymorphic. The explanation for this phenomenon lies in the evidence that it is the α/β dimer part of the TCR that is responsible for recognition and binding of the antigen presented in the context of a highly polymorphic MHC molecule on the surface of APCs. The δ , ϵ , and ζ subunits of the CD3 complex are associated with protein tyrosine kinases Lck, Fyn and ZAP-70, and are involved in transducing the signal from the receptor (Paul *et al.*, 1993).

Structure of MHC Molecules

The MHC proteins, which are essential for presenting antigens to T cells, fall into two structurally and functionally distinct classes, I and II. Class I MHC molecules (MHC I) are responsible for presentation of intracellular (primarily viral) antigens to T cells. They are expressed on the surface of most cells and consist of a 45-kDa α chain associated with a 12-kDa subunit (not encoded in the polymorphic MHC region), referred to as β_2 -microglobulin. The α chain is a type II membrane protein that consists of three extracellular domains designated $\alpha 1$, $\alpha 2$ and $\alpha 3$, a membrane-spanning domain, and a short cytoplasmic tail. The $\alpha 1$ and $\alpha 2$ domains form the binding site

for the antigen-derived peptide and for the TCR (Germain, 1994).

The overall three-dimensional conformation of a class II MHC molecule (MHC II) is very similar to that of MHC I. There are, however, certain structural differences between the two classes of MHC proteins. MHC II is expressed by professional APCs, such as B cells, monocyte-derived macrophages, dendritic cells and endothelial cells. The MHC II protein consists of a 33-kDa α chain and a 29-kDa β chain. Both the α and β subunits contain a membrane-spanning domain and are encoded in the MHC region. Class II MHC molecules are responsible for presentation of extracellular antigens (such as bacterial antigens) to T lymphocytes (Germain, 1994).

Conventional Antigens and Superantigens

T cells can only recognise conventional antigens in the context of a MHC molecule. Consequently, in order to be recognised by T cells, an extracellular antigen must first undergo internalisation and intracellular processing by an APC. The antigenic protein is endocytosed, digested by lysosomal enzymes, and the resulting short peptide is loaded onto the MHC II heterodimer. Unloaded, MHC II proteins are unstable and are eventually degraded by lysosomal proteases. The MHC II-peptide complex is then transported to the surface of the cell (Neefjes *et al.*, 1993). It is the MHC II-peptide complex that is recognised and bound by the T-cell antigen receptor (TCR). This binding involves the variable (V) and joining (J) regions of both α and β polypeptide chains of the TCR, as well as the diversity (D) region of the β chain (Germain, 1994). The term 'superantigens' (SAGs) is used to describe these unique immunogenic compounds that are able to stimulate unusually large numbers of T cells, up to 20% of the entire T-cell complement of the host (Webb and Gascoigne, 1994). In contrast to conventional antigens, these proteins, usually of bacterial or viral origin, are not internalised by APCs, do not undergo intracellular processing, and are not presented on the surface of APCs as short antigen-derived peptides (Murray *et al.*, 1995).

A schematic representation of SAG interactions with TCR and with the MHC on the antigen-presenting cell is shown in Fig. 2. In contrast to conventional antigens, intact superantigenic proteins bind to MHC II at a site distinct from the peptide-binding site (Jardetzky *et al.*, 1994). Likewise, the binding site for SAG on the TCR is different from the region involved in recognition and binding of the MHC-peptide complex (Seth *et al.*, 1994). Indeed, interaction with a superantigen involves primarily the variable region of the TCR β chain (V β) (Malchiodi *et al.*, 1995). Thus,

recognition of SAGs by T cells appears to be based mainly on the V β portion of the TCR without notable contribution of other regions of the receptor. As a result, SAG recognition by T cells is subject to a significantly lower degree of specificity, as compared with their interaction with conventional antigens, which, in turn, may explain the ability of SAGs to stimulate unusually large numbers of T cells.

Superantigenicity of Staphylococcal Enterotoxins

The SEs exert a 3-fold effect on the host organism: (1) as enterotoxins, they induce emesis and diarrhoea in humans and non-human primates (Jett *et al.*, 1994); (2) as exotoxins, they have been implicated in induction of toxic shock (Marrack and Kappler, 1990); and (3) as SAGs, they induce V β -specific T-cell stimulation (Rellahan *et al.*, 1990), followed by anergy and activation-induced cell death (AICD) by apoptosis, which, in turn, leads to immune suppression (Betley *et al.*, 1992).

The clinical manifestations of SE intoxication are associated primarily with the large-scale release of pro-inflammatory cytokines, particularly interleukin 2 (IL-2) and tumor necrosis factor α (TNF α) (Marrack and Kappler, 1990; Johnson *et al.*, 1991; Miethke *et al.*, 1992), as a result of the massive proliferation of T lymphocytes. However, *certain* SEs exert a direct effect on the target organs, particularly the kidney, thereby contributing to the dysregulation of vascular tone, which results in severe hypotension associated with SE-induced toxic shock (Chatterjee and Jett, 1992; Chatterjee *et al.*, 1995; Khullar and Chatterjee, 1995; Ionin *et al.*, 2000). Thus, it has become exceedingly difficult to fully separate the exotoxic, enterotoxic and superantigenic activities of the SEs.

Staphylococcal Enterotoxins and the T-Cell Receptor

Following the resolution of the crystal structures of some of the SEs and TCR (Swaminathan *et al.*, 1992; Malchiodi *et al.*, 1995; Fields *et al.*, 1996; Bentley *et al.*, 1997), attempts have been made to identify the regions of the toxins important for their immunomodulatory effects. In general, there is a distinct functional separation of the SE N-terminal and C-terminal domains, whereby one domain is responsible for SE interaction with TCR, while the other domain mediates SE-MHC II interaction (Kappler *et al.*, 1992; Swaminathan *et al.*, 1992; Soos *et al.*, 1993; Hurley *et al.*, 1995; Mahana *et al.*, 1995; Li *et al.*, 1998a, b), but the specificities vary between different members of the SE family. The SE-TCR interaction appears to play a pivotal role in the superantigenic

activity of SEs. Indeed, a direct correlation has been established between SE affinity for the TCR and its ability to stimulate T cells (Li *et al.*, 1998b).

It must be noted, however, that details of the SE-TCR interaction remain the subject of some controversy. For instance, it was originally reported that SEB residues in positions 60 (Asn) and 61 (Tyr) are responsible for the SEB-TCR interaction (Kappler *et al.*, 1992), yet it was later shown that the loop 60-61 mediates interaction of the toxin with some, but not all, of the human and murine SEB-specific V β TCR elements (Mahana *et al.*, 1995). Furthermore, synthetic peptide studies of enterotoxin activity reveal SE regions, previously not associated with TCR or MHC interactions, that block SE stimulation of T-lymphocyte proliferation (Harris *et al.*, 1993a; Jett *et al.*, 1994; Soos and Johnson, 1994; Hoffman *et al.*, 1996). The same non-TCR peptide region (SEB 130-160) mimics SEB apoptotic activity in cultures of human kidney proximal tubule cells (Chatterjee and Jett, 1992; Chatterjee *et al.*, 1995; Khullar and Chatterjee, 1995). This led to the hypothesis that multiple regions of the SEB molecule might play a role in *its* SE activities.

The theory that T-cell specificity of bacterial superantigens depends solely on the V β region of the TCR has also been challenged. The junctional region of the TCR β chain (J β) is required for full T-cell activation by certain SEs (Pullen and Bogatzki, 1996). Moreover, the TCR α chain variable region has also been implicated in contributing to the T-cell recognition of a superantigen (Deckhut *et al.*, 1994; Daly *et al.*, 1995). It became apparent that multiple sites on the SE and the TCR molecules contribute to the T-cell interaction with the superantigens, thus rendering the nature of TCR-SE interaction far more complex than had originally been thought.

Staphylococcal Enterotoxins and MHC

Although superantigens do not undergo internalisation and proteolytic processing by the APC, a characteristic of conventional extracellular antigens, in most cases they still require presentation by the APC in order to be recognised by the T cell. However, instead of the short antigen-derived peptide, the intact superantigenic protein binds to the class II MHC at a site different from the peptide-binding groove formed by the α/β MHC dimer (Jardetzky *et al.*, 1994).

Different members of the SE family bind to different sites on the class II MHC molecule (Chintagumpala *et al.*, 1991), and differ in their repertoire of compatible MHC molecules (Herman *et al.*, 1990). Some SEs may possess more than one MHC-binding site, thus providing a possible explanation for their stronger

MHC binding affinity (Kappler *et al.*, 1992; Soos and Johnson, 1994). Moreover, it has been shown that certain SEs have the ability to stimulate T lymphocytes in the absence of a class II MHC presentation (Avery *et al.*, 1994), leading to the classification of superantigens into two subgroups: MHC-dependent and MHC-independent.

Based on this evidence of the differential TCR and MHC binding characteristics, as well as the correlation between the TCR and/or MHC binding affinity and the superantigenic activity, it has been proposed that the SE-TCR, SE-MHC and MHC-TCR binary complexes together form a fluid ternary complex characterised by compensatory affinities, whereby weaker interactions are stabilised by the stronger ones (Seth *et al.*, 1994; Li *et al.*, 1998a b). This model of SE-TCR-MHC interaction appears to provide the best description of the structure-function basis for the SE stimulation of T lymphocytes. In summary, staphylococcal enterotoxins have strong superantigenic activity manifested by the induction of polyclonal T lymphocyte proliferation, which results in anergy and apoptosis, and leads to deletion of a large portion of the T-cell complement of the host. This effect originates in the peculiarities of the superantigen interactions with the TCR and the class II MHC molecules, namely lack of antigen processing by the APC and binding to the MHC outside the peptide-binding groove. This, in turn, results in a lower degree of TCR and MHC specificity, as compared with conventional antigens. While the evidence from structure-function studies of the SE-TCR and SE-MHC interactions is still subject of some controversy, the leading model of T-cell stimulation by SE suggests a direct correlation between binding affinities of SE, TCR and class II MHC on one hand (with weaker interactions being compensated for by the stronger ones), and the ability of the toxin to stimulate T lymphocytes on the other hand.

Renal Function and Staphylococcal Enterotoxins

Localisation in the Kidney

Evidence from various early animal models implicates the kidney in general, and renal proximal tubule cells in particular, as the major target of SEB uptake (Morris *et al.*, 1967; Rapoport *et al.*, 1967; Normann *et al.*, 1969). The SEB-induced death of rabbits can be prevented by nephrectomy after toxin administration. The rapid clearance of ^{131}I -labelled SEB from the blood in Rhesus monkeys is retarded by renal artery ligation, but it is unaffected by the pharmacological blockade of the reticuloendothelial (phagocytic)

system, suggesting that the enterotoxin is sequestered in the kidney epithelium (Rapoport *et al.*, 1967). If [^{131}I]SEB is administered intravenously to monkeys, 32% is localised to the kidney within 30 minutes, as determined by autopsy, and 97% of the radioactivity in the kidney is in the renal cortex, which is the site of proximal renal tubules (Morris *et al.*, 1967).

These observations are supported by immunofluorescence studies, which showed that sequestration of fluorescein isothiocyanate (FITC)-labelled SEB in the kidney is prevented if glomerular filtration is interrupted by ligation of the ureters (Normann, 1971; Normann and Stone, 1972). Interestingly, fluorescence was not detected in the liver of monkeys, while in rats FITC-SEB is present in both liver and kidneys. Indeed, in nephrectomised rats, the liver becomes the principal site of SEB concentration in terms of the intensity of fluorescence, which implicates the liver as an alternate site of toxin uptake in rodents (Normann *et al.*, 1969). This led to the hypothesis that renal sequestration of SEB in primates, but not in rodents, is receptor-mediated and may contribute to the differences in SEB sensitivity between rodents and primates. An example is the finding that neutral glycosphingolipid SEB receptors are present in primate kidneys but not in rodent kidneys (Khullar and Chatterjee, 1995). Neuronal binding of SEA in the gastro-intestinal tract is not demonstrable (Beery *et al.*, 1984). The potentiation by LPS of SE, and vice versa, has been demonstrated in various systems. In a rat model of LPS-induced necrosis of isolated rat renal tubular cells (RTCs), TSST-1 enhances the cytotoxic effects of LPS/lipid A. Oxidative metabolism of arachidonic acid and the generation of reactive oxygen species appears to participate in LPS/lipid A-mediated RTC death (Keane *et al.*, 1986).

The Effect of Binding to Kidney Cells

The binding of SEB, but not SEA or TSST-1, to primary cultures of human proximal tubule cells is concentration-dependent and it was deduced that the receptor is the neutral glycosphingolipid CerGal α 1 \rightarrow 4Gal (Chatterjee and Jett, 1992; Chatterjee *et al.*, 1995). SEB, but not SEA or TSST-1, increases the uptake of [^{14}C]choline into phospholipids (Khullar and Chatterjee, 1995). It has been shown that SEB binds to COS-1, an African green monkey kidney fibroblast-like cell line, which does not express MHC class II, but nevertheless the binding of SEB was saturable. The receptor is a membrane protein of approximately 85 kDa (Rogers *et al.*, 1995). SEC1, SEC2 and SEC3 also bind to this protein, but SEA, SED, SEE and TSST-1 do not bind. Truncated fusion

new paragraph
With 70% of injected SEB
eventually accumulating in the proximal tubule
within 2 hrs

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proteins and use of structure-specific antisera showed that residues at or near the C-terminus of SEB are critical for binding to the 85-kDa protein (Rogers and Zhang, 1997). Analysis of changes in gene expression by primary cultures of renal proximal tubule epithelial cells shows specifically altered expression of several genes responsible for the regulation of vascular tone. These genes were not previously known to be associated with SE-induced illness and they, or their encoded proteins, are potentially new therapeutic targets for the treatment of SE-induced lethal shock (Ionin *et al.*, 2000). *TSST-1*

Oral challenge to rhesus monkeys with SEB produces few changes in cardiorenal function, but intravenous challenge causes hypotension, tachycardia, increased total peripheral and renal vascular resistance, and a decrease in cardiac and renal functions. The early renal depression is not associated with hypotension, but all measured renal functions other than the extraction ratio of polycyclic aromatic hydrocarbons correlate positively with decreased blood pressure in the later phase of SEB toxemia, and renal impairment has been thought to contribute to death during SEB enterotoxaemia (Liu *et al.*, 1978a, 1978b). There is an increase in the nitric oxide synthase (NOS) in response to TSST-1 in cultures of murine monocyte-macrophage cells (J744.2) and pig kidney epithelial cells, which is due to concentration-dependent elevations of cGMP. Hydrocortisone, but not indomethacin, prevents the generation of NO₂ and the increase in cGMP levels, and this is partially reversed by co-incubation with RU 486, an antagonist of glucocorticoid receptors (Zembowicz and Vane, 1992).

Renal Failure in Toxic Shock Syndrome

Renal failure is part of the clinical picture of fully developed TSS. For example, a 73-year-old woman developed acute renal failure due to TSS, with negative cultures of blood and cerebrospinal fluid, but MRSA that produced TSST-1, SEB and SEC was isolated from her pharynx and vagina (Fujisawa *et al.*, 1998). Similarly, a 21-year-old man with colitis and sepsis developed acute renal failure due to infection with an MRSA that produced TSST-1. On day 8 renal biopsy showed severe acute interstitial nephritis with medullary inflammatory cell infiltration, but without glomerular changes. Many lymphoid cells were present in the interstitium, and most of the infiltrating cells were small monocytes; over 70% of T cells were Vβ2-positive (Owada *et al.*, 1999). Another patient was a 71-year-old man with pneumonia, who developed proteinuria and renal failure and from whom an MRSA was isolated that produced SEB, SEC and TSST-1.

Raised levels of IgG and IgA and various cytokines were present and a renal biopsy showed mesangial proliferative glomerulonephritis with deposits of IgG, IgA and C3 along the capillary walls (Yoh *et al.*, 1997).

In the case of a 31-year-old woman with menstrual-related TSS, organisms isolated from vagina, nose and trachea produced enterotoxins A and F. She presented with shock, a high fever, skin eruption, pulmonary interstitial oedema and acute renal failure that required dialysis, and residual renal failure was still present 4 months later (Bouletreau *et al.*, 1984). Cases of sudden infant death syndrome (SIDS) from several different countries have also been studied. In approximately 50% of such infants, TSST-1, SEA, SEB and SEC1 could be detected by ELISA, but toxins were present in only 16% of infants who died from non-SIDS related causes (Zorgani *et al.*, 1999).

Interaction with Endothelial Cells

SE-induced illness is associated with a variety of clinical manifestations, of which the most serious is acute respiratory distress syndrome (ARDS). Histological examination of monkeys exposed to SEB showed widely distributed pulmonary oedema. The adventitia of pulmonary vessels were infiltrated by lymphocytes, macrophages and some neutrophils. Numerous large lymphocytes with occasional mitotic figures were present in pulmonary vessels, and often occluded the alveolar capillaries. The alveolar septal interstitial spaces were expanded by oedema (Mattix *et al.*, 1995). Recruitment of neutrophils to the alveoli and airways has been demonstrated when SEA or SEB is administered intravenously to rabbits (Wagers *et al.*, 1998) and to mice (Tessier *et al.*, 1998). The chemo-attractants involved in this recruitment may include IL-8 and/or ICAM-1 (Xu *et al.*, 1994).

SEA induces injury to normal human lung microvascular endothelial cells (HMVEC-L) and is attributable to SE-induced cytokine production by polymorphonuclear cells (PMN) (Fujisawa *et al.*, 1998). Similarly, SEB-stimulated LAK cells induce glomerular endothelial cell injury (Seprenyi *et al.*, 1997). Other studies suggest that contributors to endothelial damage and to multi-organ failure caused by SEs may include platelets, which show the typical release reaction of inflammatory mediators and serotonin, in response to SEB as well as to LPS (McKey *et al.*, 1997).

SEB has direct effects on primary cultures of pulmonary artery endothelial cells in the absence of effector cells or their products. Barrier function is disrupted and the intracellular junctions show SEB-induced tyrosine phosphorylation, and inhibitors

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9A

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There are probably renal receptors for the SEA family of enterotoxins in view of the observations that SEA-exposed individuals have been characterized as having residual kidney-related problems (Carmo et al 2001)

of protein tyrosine kinase block loss of barrier function in these cells (Riesbeck *et al.*, 1998). TSST-1 directly disrupts barrier function and leakage occurs in porcine aortic endothelial cells without priming by lymphoid cells or cytokines (Lee *et al.*, 1991).

Attempts have been made to understand the course of SE induction of respiratory distress. Human umbilical vascular endothelial cells (HUVEC) stimulated with IFN γ , or with SE-primed T cells express HLA class II molecules after some days. At that time, binding was observed of TSST-1 (Araake *et al.*, 1991), SEA (Kushnaryov *et al.*, 1989; Uchiyama *et al.*, 1990; Riesbeck *et al.*, 1998) and SEB (Krakauer, 1996). In the case of unprimed HUVECs, *Staph. aureus* but not staphylococcal enterotoxins could act as primary stimulus for endothelial cells to induce production and release of cytokines (Soderquist *et al.*, 1998). Another approach involved induction of cell adhesion molecules correlated with the binding of SE (Srinivas *et al.*, 1998). This model system should be applicable to bacterial infections in which toxin secretion occurs over an extended time period. SEs disappear from circulation by 30–60 minutes post exposure. The model does not, therefore, appear to be an appropriate model to study toxin-induced illness in which the organism is not present, such as in food poisoning or toxin inhalation, since HUVECs require days of priming before the effects of SE are seen.

Skin Disorders and Staphylococcal Enterotoxins

Skin colonisation with *Staph. aureus* occurs in numerous skin disorders, including atopic dermatitis (AD), Kawasaki's syndrome, scalded skin syndrome, psoriasis and others (Noble *et al.*, 1998). Depending on the disorder, 65–80% of the staphylococci produce one or more of the SEs (Leung *et al.*, 1995; Norris and Leung, 1997) and absorption of the toxin does not differ through normal or atopic skin. The inflammation is thought to be directly related to actions of secreted SEs. The up-regulation by SEs of skin-homing receptor and cutaneous lymphoid antigen may promote T-cell localisation to the skin and an abundance of macrophages appear in the lesions. The patterns of cytokine mRNA in these patients are consistent with TH2 phenotype (Norris and Leung, 1997).

Auto-immune Disorders and Staphylococcal Enterotoxins

Rheumatoid Arthritis

SEs promote production of antigen-specific antibodies in models of rheumatoid arthritis (RA) (He *et al.*, 1992) and they induce rheumatoid factor (RF), which

is an auto-antibody directed against the Fc portion of immunoglobulin. RF is present in 70% of rheumatoid arthritis cases and it is associated with a serious prognosis, including cutaneous vasculitis, interstitial fibrosis and Felty's syndrome (Schiffenbauer *et al.*, 1997). In a mouse model of RA, injection of type II collagen leads to severe chronic arthritis 4–5 weeks later (Knudsen *et al.*, 1997). The injection with *Mycoplasma arthritidis* or SEB (but not SEA) into convalescent mice whose arthritis had largely resolved after 130–200 days leads to a rapid, severe flare-up of the arthritis (Knudsen *et al.*, 1997). Sequence comparisons between *Mycoplasma arthritidis* mitogen and SEB show an alignment at residues 38–82 (Kappler *et al.*, 1992) and with C1 at residues 127–139 (Hoffman *et al.*, 1996). RA patients have increased IgM against SEB (Origuchi *et al.*, 1995a, 1995b), and anti-SEB but not anti-SEA is present in RA patients (Knudsen *et al.*, 1997). The disorders classified as reactive arthritides occur after genito-urinary or gastro-intestinal bacterial infections (Schiffenbauer *et al.*, 1997). On the whole, these findings suggest that the SEs can lead to the onset of auto-immunity that results in rheumatoid arthritis in predisposed individuals.

Multiple Sclerosis

Multiple sclerosis (MS) is thought to be an antigen-specific immunity to central nervous system proteins such as myelin, proteolipid protein, etc. Auto-immune experimental allergic encephalomyelitis (EAE) induced in PL/J mice by immunisation with myelin basic protein (MBP) is an animal model of MS. On the administration of SEB or SED to mice that had recovered from an episode of EAE, the mice developed a clinical relapse of the disease (Matsumoto and Fujiwara, 1993; Brocke *et al.*, 1996; Schiffenbauer *et al.*, 1997). Although SEA does not activate V β 8+ T cells, it induces relapse of EAE in mice (Schiffenbauer *et al.*, 1997). Development of EAE in naïve mice occurs on the transfer of SEB-activated, MBP-reactive T lymphocytes (Racke *et al.*, 1994).

Kawasaki Disease

Kawasaki disease (KD) is an acute febrile illness with persistent fevers, redness of lips, oral mucosa and a polymorphous skin rash that lasts for 1–2 weeks. It is the most common cause of acquired heart disease in children in Japan and the US (Norris and Leung, 1997). KD occurs most commonly in Japanese children and may occur in epidemics. It has been shown that 70% of KD patients harbour SE-producing bacteria (Leung *et al.*, 1993b) and that the pattern of T-cell expansion in these patients suggests superantigen activation (Curtis *et al.*, 1994; Curtis *et al.*, 1995).

incorrect reference, would be Campbell *et al.* (1997)

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Auto-immune Lung Disease

Auto-immune diseases, such as idiopathic pulmonary fibrosis, have been suspected of involving superantigen stimulation. A mouse model has been developed in which auto-immune mice develop interstitial pneumonia on the intratracheal administration of SEB or SEA, but not protein A (Shinbori *et al.*, 1996). In a similar mouse model, SEB induces cytokine production in the bronchoalveolar lavage (BAL). An immunosuppressant (FK506), but not corticosteroid, inhibits SEB-induced T-cell expansion in BAL fluid and increases cytokine and chemokine production in the bronchoalveolar space of SEB-treated mice. Furthermore, transfer of T cells from auto-immune mice stimulated with SEB into severe combined immunodeficiency (SCID) mice gives rise to interstitial pneumonia. This suggests that superantigen-reactive T cells in the bronchoalveolar space may trigger the development of interstitial pneumonia in this mouse model (Fujiki *et al.*, 1999). In a comparison of lung fluid and peripheral blood, patients with hypersensitivity pneumonitis or sarcoidosis showed T-cell subsets typical of SEs, and with recovery these subsets disappeared (Trentin *et al.*, 1997).

Other Auto-immune Disorders

Auto-immune disorders in which SEs may be involved include insulin-dependent diabetes mellitus, Wegener's granulomatosis and psoriasis (Schiffenbauer *et al.*, 1997). A substantial body of circumstantial evidence suggests that SE may play a role in auto-immunity and it appears likely that SEs can exacerbate a pre-existing auto-immune tendency (Knudsen *et al.*, 1997).

Antibody Characteristics and Various Disorders

Many adults have significant anti-SE antibody titres (Bergdoll *et al.*, 1973). We have noticed that high-titre antisera decrease SEB-induced lymphocyte proliferation to about 30% of control (Jett *et al.*, 1994). The residual activity could not be blocked even by addition of another source of antisera. It has been reported that antisera from only few specific individuals completely block SE mitogenic activity. This may explain why individuals have repeated bouts of SE intoxication. Intravenous administration of commercial immunoglobulin preparations is effective in the treatment of TSS, especially in the normalisation of blood pressure (Barry *et al.*, 1992).

Attempts have been made in a number of diseases to understand how they may relate to exposure to SEs. Bunikowski *et al.* (1999) investigated the prevalence of

circulating anti-SEA, SEB-specific IgE antibodies in children with atopic dermatitis and found that 34% had antibodies to SEB or SEA. These children had significantly more severe atopic dermatitis and levels of specific IgE to food and air allergens. The disease severity correlated to a greater extent with the presence of SEA/SEB-specific antibodies than with total serum IgE levels. Density of colonisation with superantigen-secreting *Staph. aureus* was higher in the superantigen IgE-positive group. The majority of these children had repeated episodes of superficial *Staph. aureus* skin infections.

As described previously, antibody levels to SEB in rheumatoid arthritis patients correlate remarkably to the disease and present a pattern different from that in matched controls (Origuchi *et al.*, 1995a, 1995b). Similarly, a higher percentage of rheumatoid arthritis patients are carriers of enterotoxigenic staphylococci and also have increased antibodies to SE than controls (Tabarya and Hoffman, 1996).

Children have frequent staphylococcal infections, and many lack antibody to TSST-1 but, surprisingly, the incidence of TSS is low in these children. In one study, 52 children had infections with toxin-producing strains and none had TSS. Protective antibodies are high in newborns, of whom 80% have protective antibody titres, they decline until 2 years of age and then gradually rise (Jacobson *et al.*, 1987). Isolates from children are more likely to be TSST-1-positive than those from adults (Jacobson *et al.*, 1989).

Mediators of Staphylococcal Enterotoxin-Induced Illness

Cytokines

The functional diversity of cytokines, and their subset of immunity and inflammation modulators, known as lymphokines, are reviewed by Meager (1998). In contrast to classical hormones, cytokines are believed to act both in a paracrine manner, acting on neighbouring cells, and in an autocrine manner, acting on the secreting cells themselves. Furthermore, unlike hormones, cytokines often exert mitogenic effects directly on their target cells. In addition, many cytokines possess a wide range of frequently overlapping biological activities (Meager, 1998). These may, in part, account for the unique role a number of cytokines play in both cellular and humoral responses of the host to infectious agents. For example, the interleukins IL-1, IL-2 and IL-6, the interferons IFN α and IFN γ (type II interferons), and the members of the TNF family are widely implicated in mediating

inflammatory responses, including those associated with systemic shock induced by Gram-negative and Gram-positive bacterial toxins, as will be described below (Miethke *et al.*, 1992; Granovitz, 1993; Granowitz *et al.*, 1993; Dinarello, 1997).

The notion that cytokines are involved in the mediation of septic shock is based on several categories of observations, including those obtained via (1) cytokine injection into experimental animals, (2) cytokine inhibitor treatment of animals injected with bacteria or bacterial toxins, (3) the pharmacokinetic studies in animals and humans, (4) the cytokine analysis of the serum from septic shock patients, and (5) the clinical trials with specific cytokine inhibitors (Wagge and Steinshamn, 1997). Animal models show a remarkable consistency in the endotoxin-induced cytokine profile in different species. Endotoxin raises levels of TNF α , IL-1, IL-6 and IL-8, and IFN γ (Redl *et al.*, 1993). Cytokine and anti-cytokine treatment of animals has confirmed the involvement of these molecules in mediation of septic shock (Ohlsson *et al.*, 1990; Doherty *et al.*, 1992). In the case of human models of endotoxin-induced shock the results are very similar to those obtained in animals. They show elevation of TNF α , IL-1, IL-6 and IL-8 levels within a few hours after exposure to endotoxin, followed by a decline to undetectable levels (Martich *et al.*, 1991).

Interestingly, while pro-inflammatory cytokine involvement in septic shock has been well established (see above), clinical trials of anti-cytokine therapies have yielded disappointing results (Table 1). Anti-TNF α antibody treatment does not increase patient survival (Cohen and Carlet, 1996; Fisher *et al.*, 1996; Abraham *et al.*, 1997, 1998; Opal *et al.*, 1997), and in some cases during phase II clinical trials the result is adverse (Abraham *et al.*, 1997, 1998). Major phase III clinical trials conducted in the mid-1990s in the US, Canada, Europe and South Africa showed no statistically significant differences in the 28-day mortality rates, although in patients with shock a general trend to improvement was observed (Fisher *et al.*, 1996; Opal *et al.*, 1997). The possible explanations for the discrepancy between the animal model results and those observed in the clinical trials include (1) the heterogeneity of the toxic shock and related syndromes in terms of aetiology and clinical manifestation; (2) involvement of other cytokines in mediation of shock resulting in circumvention of anti-TNF treatment or anti-IL-1 treatment; and (3) contribution of other soluble factors, tissues, organs and systems to the progression of the shock syndrome (Chatterjee *et al.*, 1995; Khullar and Chatterjee, 1995; Campbell *et al.*, 1997; Wagge and Steinshamn, 1997). Research is clearly required to develop new complex

animal models to cover the spectrum of clinical situations, and to investigate other possible mechanisms involved in mediation of septic shock.

Staphylococcal Food Poisoning

Work with mutant SE proteins suggests that their 'superantigenicity' does not correlate with the emetic response in monkeys (Harris *et al.*, 1993b; Hovde *et al.*, 1994). In mice, however, SEB given intragastrically induces mucosal T-cell expansion and up-regulation of cytokine mRNA (Spiekermann and Nagler-Anderson, 1998). In human fetal gut explant cultures SEB produces severe villous atrophy and crypt cell hyperplasia. IL-10 down-regulates mucosal T-cell activation, metalloproteinase production, and loss of extracellular matrix, and prevents mucosal damage in the gut (Pender *et al.*, 1998).

Arthritis and Dermatitis

A role for bacterial superantigens in several chronic inflammatory diseases has been associated with an imbalance of the T helper cell subsets and their cytokine production (Weber *et al.*, 2000). Patterns of synovial and systemic cytokine mRNA expression in mice with superantigen-mediated SEA-induced arthritis shows up-regulation of TNF α and IL-1 β mRNA and decrease in anti-inflammatory IL-4 and IL-10 mRNA expression (Zhao *et al.*, 1996). Similar results are seen with TSST-1 (Zhao *et al.*, 1995). The role of IFN γ in the regulation of host-resistance to SE-induced arthritis has been studied with IFN γ receptor-deficient mice, which developed a more severe form of the disease and had a higher incidence of lethal shock in early stages (Zhao *et al.*, 1995).

SE toxins that colonise skin lesions on patients with chronic atopic dermatitis induce the production of GM-CSF, resulting in inhibition of monocyte-macrophage apoptosis, and thus extending the inflammation (Bratton *et al.*, 1999). SEA, B, C1 and C2 each induce strong ICAM-1 expression in organ-cultures of human keratinocytes. TNF α and protein are over-produced in keratinocyte cultures, while IL-1 β and IL-1 α are expressed at the mRNA level. This increases cell adhesion molecule expression, which is likely to be involved in the induction of refractory eczematous lesions in atopic dermatitis (Matsunaga *et al.*, 1996). Superantigens are potent inducers of several pro-inflammatory cytokines, such as IL-1 and TNF α in the antigen-presenting cells. In atopic dermatitis the lesions increase in response to SEB, since IL-4 production by mast cells is decreased (Ackermann *et al.*, 1998). Skin T cells respond to SEB by producing IL-2, IL-5, IL-13 and IFN γ , but not IL-4, and the

Table 1 Clinical trials with immunomodulating agents in patients with sepsis (1996–1998)

Agent	Phase	Year	No. patients	28–30 day all-cause mortality	Results	Reference
Anti-TNF α mAb	III	1996 Intersept	553	No significant improvement. In the lower-dose group, reduction by 14.5%	Other findings More rapid reversal of shock; significant delay in the time of the onset of organ failure	Cohen and Carlet, 1996
Anti-TNF α mAb	III	1998 Intersept	1879	No significant improvement. 5.8% reduction	Significant decrease in the frequency of coagulopathy	Abraham <i>et al.</i> , 1998
sTNFR p75-IgG	II	1996	141	No significant improvement	Increase in mortality in the subgroup that received higher doses	Fisher <i>et al.</i> , 1996
sTNFR p55-IgG	III	1997	498	No significant improvement	Significant improvement of 28-day survival of patients with severe sepsis	Abraham <i>et al.</i> , 1997
IL-1ra	III	1997	696	No significant improvement	A better resolution of organ failures	Opal <i>et al.</i> , 1997
Liposomal PGE1	III	1996	26 ARDS	No significant improvement	Improvement in lung compliance; earlier removal from mechanical ventilation	Abraham <i>et al.</i> , 1996
Ibuprofen	III	1997	455	No significant improvement	Delay in the day of the onset of organ failure	Bernard <i>et al.</i> , 1997
Bradykinin antagonist	II	1997	504 (SIRS)	No significant improvement	Significant improvement of 28-day survival of patients with Gram-negative infections	Dhainaut <i>et al.</i> , 1994
Antithrombin III	III	42	42	No significant improvement	A better resolution of organ failures; a lower incidence of new organ failures	Eisele <i>et al.</i> , 1998

Reprinted from Karima *et al.* (1999), with permission from Elsevier Science. ARDS, acute respiratory distress syndrome; IL-1ra, IL-1 receptor antagonist; INTERSEPT, International Sepsis Trial Group; mAb, monoclonal antibody; NORASEPT, North American Sepsis Trial Group; PGE1, prostaglandin E1; SIRS, systemic inflammatory response syndrome; sTNFR, soluble tumour necrosis factor receptor.

response is significantly lower than that produced by skin T cells from non-allergic atopic dermatitis patients (Akdis *et al.*, 1999a, b).

Toxic Shock Syndrome Due to Staphylococcal Enterotoxins

Cytokines have been widely studied in relation to the lethal shock induced by LPS and the SEs. An extensive literature and many excellent reviews on the subject have been published that describe the involvement of cytokines in LPS-induced shock (Wagge and Steinshamn, 1997; Karima *et al.*, 1999). The complexities of cytokine generation in response to SEs are intertwined with various signal transduction pathways, which will be considered below. Of the pro-inflammatory cytokines, TNF α and especially its synergistic action with IFN γ has been the subject of numerous studies. Indeed, mouse models of SE toxicity, in which mice are compromised with high doses of D-galactosamine (D-Gal) or LPS, have shown that they respond to the SEs by producing massive amounts of TNF α (Bette *et al.*, 1993; Miethke *et al.*, 1993, 1994; Stiles *et al.*, 1993). Furthermore, in the D-Gal mouse model, anti-TNF α effectively abrogates lethality while antisera to IFN γ or IL-1 do not (Nagaki *et al.*, 1994; Matthys *et al.*, 1995). Hepatic apoptosis induced by TNF α , followed by necrosis, may represent a general pathological mechanism of T cell shock models in which D-galactosamine-sensitised mice are used (Gantner *et al.*, 1995, 1996, 1997). A murine chimaera model also shows an increase in pro-inflammatory cytokines in response to SEB (Yuan *et al.*, 2000). As was pointed out above (Table 1), the remarkable success in blocking lethality in mice by targeting TNF α cannot be reproduced in primates, nor is it successful in the treatment of lethal shock in humans (Cohen and Carlet, 1996; Fisher *et al.*, 1996; Abraham *et al.*, 1997, 1998; Opal *et al.*, 1997). This failure does not detract from the role of TNF α as an important mediator in lethal shock. The timing of TNF α production may be rather brief, as has been seen in non-human primates and, clearly, many concurrent events contribute to toxic shock.

The cellular source of the various cytokines has been studied extensively and correlates with SE-induced proliferation in T cells. However, in monocyte cell cultures, SEA, SED and SEE, each of which causes dimerisation of MHC II molecules on monocytes, induces gene expression of TNF α and IL-1 β (Al-Daccak *et al.*, 1998), while cultures of human peripheral blood mononuclear cells (PMBC) exposed to high concentrations of SE show TNF α production equally by both monocytes and T cells (Andersson

et al., 1989; Andersson and Matsuda, 1989; Fischer *et al.*, 1990). Under nearly identical conditions, low concentrations of SEB stimulate TNF α production by T lymphocytes, rather than from monocytes. The process depends on protein kinase C and also involves phosphatidylinositol-3 kinase (Yan *et al.*, 1999). The timing of TNF α production may be very important in potential treatments, and its production in non-human primates has been shown to take place approximately 1.5–3 hours after challenge with SEB. In addition to TNF α , IL-1 secretion by monocytes is also a major consideration and the balance between IL-1 β and its antagonist, IL-1ra, is seriously upset by SEA stimulation. These alterations in their balance seem to mediate the inflammatory response (Al-Daccak *et al.*, 1994).

Superantigens activate large families of T cells, based on expression of the V β chain of the T-cell receptor. As a result, the reactive cells proliferate, secrete high levels of inflammatory cytokines, and ultimately become anergic and/or die by apoptosis. Superantigens cause deletion of specific T cells *in vivo*. As in the case of monocytes, SEB caused the secretion of a variety of pro-inflammatory cytokines (Gonzalo *et al.*, 1994b) and mRNA coding for these cytokines (Koide *et al.*, 1996). Another pro-inflammatory cytokine, IL-6, is massively produced in response to SEA (Andersson *et al.*, 1989; Andersson and Matsuda, 1989) and SEB (Mendis *et al.*, 1998). Macrophage inhibition factor is a likely intermediate in TNF α production by LPS or SEs in the D-Gal mouse model (Bozza *et al.*, 1999) and in cell cultures (Calandra *et al.*, 1998). Production of TNF β , also called lymphotoxin, is a well-characterised response to stimulation by SE (Guo *et al.*, 1999).

In addition to the pro-inflammatory cytokines produced in response to SEs, some workers have shown a second wave of release of the anti-inflammatory cytokines IL-4 and IL-10, which act to down-regulate IFN γ and TNF α (Gonzalo *et al.*, 1994b). The value of IL-10 in blocking certain actions of SEs has also been described (Bourrie *et al.*, 1996; Hoiden and Moller, 1996; Sundstedt *et al.*, 1997) and decreased production of IL-10 and IL-12 in peripheral blood mononuclear cells (PBMC) correlates with their decreased proliferation when obtained from elderly patients (Castle *et al.*, 1999). SEB is much more lethal to IL-10 knockout mice than to wild-type mice and concomitantly it produces an increase in pro-inflammatory cytokines (Hasko *et al.*, 1998). On the whole, these results suggest that IL-10 plays an important immunoregulatory role in the response to a superantigenic stimulus by modulating the shock-inducing inflammatory response.

(A) In SE exposure, elderly patients most often die. Lethality is seen among elderly (>70 years old) and very young patients (<3 years old).

Anti-cytokine Therapy

As we have seen, clinical trials that target various cytokines have not been successful (Table 1). Various drugs that have been studied for their usefulness in treatment of lethal shock have also been used to counteract cytokines. In a murine model, SEB is blocked by high doses of retinol during the early phase (< 6 hours), and by glucocorticoid receptor blockade with RU-38486 at a later phase (> 24 hours), but reversal of the order of the drugs is ineffective (Gonzalo *et al.*, 1994a).

Dexamethasone is generally ineffective (Weng *et al.*, 1999), but in rabbits, pre-treatment with indomethacin or dexamethasone blocks SEA-induced fever and the increased circulating levels of IFN γ , TNF α and IL-2 (Huang *et al.*, 1997). Ebselen protects D-Gal-sensitized mice from liver injury by SEB and, at the same time, the release of the pro-inflammatory cytokine TNF α is down-regulated, while the circulating amount of the anti-inflammatory cytokine IL-10 is increased (Tiegs *et al.*, 1998). Linomide blocks cytokine production *in vitro* (Arad *et al.*, 1996). SEA together with an anti-SEA antibody actually increase TNF α and IL-6 secretion by macrophages, and this is thought to be due to cross-linking macrophage MHC I receptors (Wright and Chapes, 1999). Targeting of the NOS mechanism has been successful with aminoguanadine (Won *et al.*, 2000) and niacinimide (LeClaire *et al.*, 1996).

Signal Transduction Pathways

Multi-cellular organisms have developed highly efficient regulatory networks through molecules that function as signal transducers and that orchestrate cellular responses to external stimuli. Signal transduction pathways are hierarchical cascades that originate at the cell membrane with the embedded receptors for the appropriate effector molecules, such as mitogens, growth factors, certain hormones, toxins and many other types of bioactive molecules. By way of adapter proteins and exchange factors the cell surface receptors for these effector molecules initiate a myriad of options for amplifying and/or modifying the output signal. Ultimately the signal is transmitted to the nucleus, where it leads to a change in gene expression and initiation of the effector-specific cellular process, such as proliferation. Transmission of signals from the cell membrane to the nucleus occurs principally by conversion of a series of proteins to their active state on phosphorylation by kinases. Many of the newly activated proteins are, themselves, kinases that phosphorylate numerous other proteins, and greatly amplify the signal. Molecules such as guanosine triphosphate

(GTP) and intracellular calcium levels also play important roles, interdigitating in many cases with the kinases. A variety of stimuli generate intracellular responses that converge on various kinase pathways (Takakawa *et al.*, 1998). The well-regulated transduction of these signals is crucial for normal cell behaviour, while aberrant signalling often leads to diverse pathological consequences.

The Signalling Cascade

Figure 3 is a schematic diagram that attempts to integrate some of the important molecules that participate in signal transduction, with a focus on the pathways involved in SE mitogenic actions on lymphocytes. Many of the stimulators of cell proliferation activate *ras*, which binds GTP and in turn activates *raf* and MAP kinase (MEK). Mitogen-activated protein kinase (MAPK) is a serine/threonine kinase, which plays a crucial role in cell proliferation and differentiation, and is a widely utilised pathway for signal transmission by diverse agents including growth factors, mitogenic proteins and even enterotoxin action on lymphocytes (Cobb *et al.*, 1991; Blenis, 1993; Das and Vonderhaar, 1996). It activates a wide variety of target proteins, including transcription factors which control gene expression (Cobb *et al.*, 1991; Nishida and Gotoh, 1993). In contrast, two novel members of MAPK-related enzymes exert opposite activities; these are the stress-activated protein kinase (SAPK) or c-Jun N-terminal kinase (JNK) and p38/MPK2. These two signalling kinases are activated in response to stress-causing agents, such as UV radiation, actinomycin D or ceramide, and they lead to cell cycle arrest and apoptosis. The Janus kinases (Jak)/signal transducers and activators of transcription (STAT) pathway is activated by numerous cytokines and growth factors and provides another signal targeting transcription factors. There is no evidence that this pathway is directly linked to the TCR/MHC II receptors. As will be discussed in more detail below, protein kinase C (PKC) shows widespread action in response to SE exposure and there is an interdependence between PKC, the phospholipases and eicosanoids (arachidonic acid metabolites). The mode of signal transduction for staphylococcal enterotoxins is important in order to understand the multifunctional roles of SE as superantigens. Most signal transduction studies have been carried out with lymphoid cells, but many important signalling details also have been established with other cell types (see below).

Kinases Involved with TCR/MHC II

Initial Kinase Actions The staphylococcal enterotoxins bind to MHC class II molecules and stimulate polyclonal T-cell populations on the basis of the

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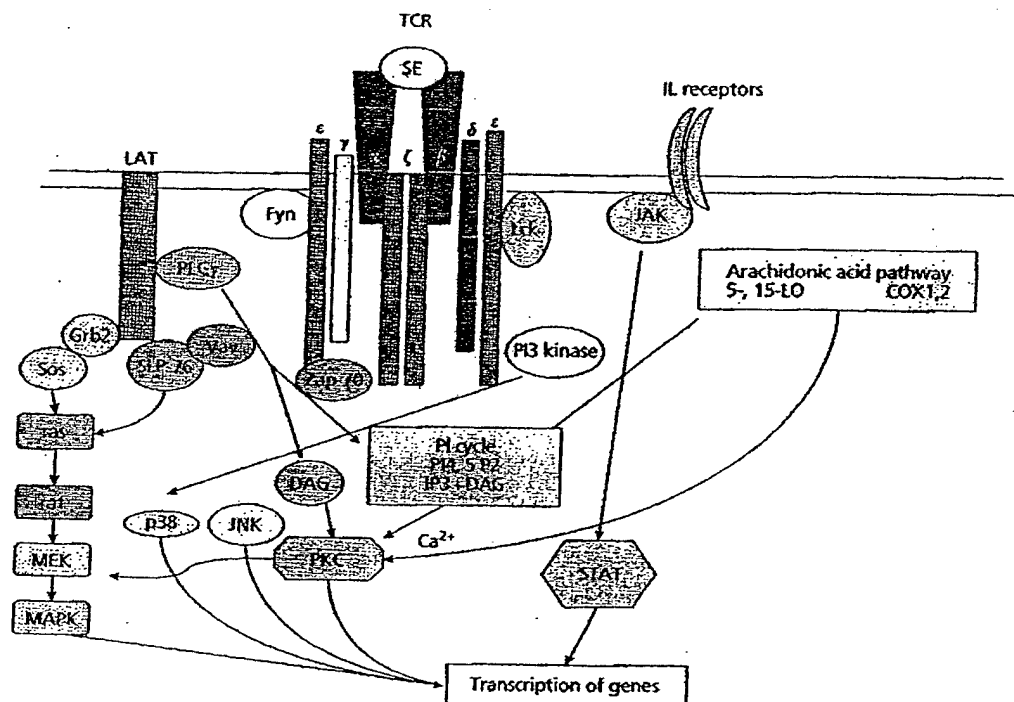


Fig. 3 A relational sketch showing the interactions of various kinases and related signalling molecules involved in staphylococcal enterotoxin-induced signal transduction cascades. The centre structure indicates staphylococcal enterotoxin (SE) binding to the T-cell receptor (TCR). The kinases ZAP-70, Lck and Fyn are recruited to initiate phosphorylation reactions.

expression of responsive TCR V β domains. Since TCR and MHC class II molecules do not have intrinsic tyrosine kinase activity, they must recruit various other kinases to transduce their signals. Tyrosine phosphorylation of TCR subunits is an initial step, along with recruitment and tyrosine phosphorylation of the protein tyrosine kinase ZAP-70 (Niedergang *et al.*, 1995, 1998). One of the earliest biochemical events detected after TCR stimulation is the activation of the Src family protein tyrosine kinases, Lck and Fyn, and, as a consequence, the phosphorylation of numerous substrates, including several TCR-CD3 subunits. CD2, an adhesion molecule on the surface of T cells, has a relatively long cytoplasmic tail that associates with Lck and Fyn (Migita *et al.*, 1995). Down-modulation of CD2 on T cells results in diminished proliferative capacity and decreased IL-2 production (Fortner *et al.*, 1998). Mice with down-modulated CD2 have decreased responses to SEB. (This may be explained by the fact that the cytoplasmic tail binds ZAP-70; SEA or SEB consistently stimulate ZAP-70 tyrosine phosphorylation (Kanner *et al.*, 1995).)

Phosphoinositide Cycle During the initial binding of SE to MHC II/TCR, protein tyrosine kinase activity increases at TCR-CD3 complexes (Niedergang *et al.*, 1998), and tyrosine phosphorylation of phospholipase C- γ (PLC- γ) occurs in T cells and in antigen-presenting cells (Conroy *et al.*, 1995). The TCR complex is thought to activate PLC- γ through a specific transmembrane adapter protein, LAT (linker for activation of T cells) (Schraven *et al.*, 1999). The activated PLC- γ cleaves phosphatidylinositol 4,5-bisphosphate to release two potent signalling molecules, inositol 1,4,5-trisphosphate (IP3), which increases calcium mobilisation, and diacylglycerol (DAG), a potent stimulator of protein kinase C (PKC). When peripheral blood mononuclear cells are stimulated with TSST-1 or SEB, the major cell producers of TNF α are T cells. PI-3 kinase regulates TNF α production in TSST-1- or SEB-treated cells and inhibitors of PI-3 kinase block TNF α production (Ramirez *et al.*, 1999; Yan *et al.*, 1999).

Protein Kinase C Activation Many workers have identified protein kinase C (PKC) involvement in

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numerous effects induced by SEs. We have shown PKC dependence in the SEB-induced appearance of cell surface adhesion molecules; TNF α , leukotriene generation and proliferation of lymphoid cells was blocked by its inhibitors (Yan *et al.*, 1999). Alteration of levels of DAG, a potent activator of PKC, by treatment with inhibitors of phospholipase C or DAG kinase, also altered SEB-induced TNF α production (Yan *et al.*, 1999).

By means of a highly selective PKC inhibitor, it was shown by Tchilian *et al.* (1996) that PKC activation is essential for the regulation of SEB-induced cell death. TSST-1 utilises PKC for signal transduction in monocytes (Trede *et al.*, 1994). Stimulation of lymphoid cells by SE results initially in proliferation, but eventually apoptosis is induced, an apparently contradictory situation. In fact, in contrast to the traditional proliferative signals just described, another series of signals is initiated by TNF α , which binds to the so-called 'death domain' of the p55 TNF α receptor, and this in turn leads to activation of phosphatidylcholine-specific phospholipase C (PC-PLC). This results in massive DAG release, and DAG is a potent stimulator of PKC (Machleidt *et al.*, 1996). It is of interest to note that this may lead to a second, indirect wave of PKC activation, since it would occur at the time of TNF α release and be a result of its action on the cells.

Protein Tyrosine Kinase Both protein tyrosine kinase (PTK) and PKC play essential roles in HLA class II molecule-mediated signal transduction elicited by SEB, and PTK may precede PKC activation in the signalling pathway (Matsuyama *et al.*, 1993). TSST-1 also induces tyrosine phosphorylation in human monocytes (Scholl *et al.*, 1992). There is no doubt that PTK activation is involved in important aspects of SE stimulation, however, in our experience, inhibitors of PTK do not block SEB-stimulated lymphocyte proliferation. In contrast, herbimycin and genistein, potent inhibitors of protein tyrosine kinases, each protect against SEB-induced cytotoxicity, barrier dysfunction, and intercellular gap formation in pulmonary endothelial cells (Campbell *et al.*, 1997). Another set of SEB-induced responses that involve PTK include increased expression of the IL-12/p40 gene in macrophages, which leads to activation and nuclear translocation of nuclear factor- κ B (NF- κ B). Inhibition of PKC or PKA activation results in suppression of these activities (Du and Sriram, 2000).

Interrelationships Between Kinases

PI-3 kinase also activates the mitogen-activated protein kinase (MAPK) family, and the p38 MAPK is also

involved in control of TNF α translation in human macrophages. In T cells, the p38 MAPK inhibitor SB 203580 significantly decreases the secretion of TNF α but not its mRNA, but combined use of PI-3 kinase with p38 inhibitors has an additive effect, which completely blocks TNF α secretion. This suggests that the PI-3 kinase and p38 MAPK signalling pathways are acting on TNF α translation independently of each other.

MAP Kinases The MAPKs are serine/threonine protein kinases that transduce signals originating from extracellular events, such as cell surface receptor engagement, culminating in the regulation of a cellular response, such as growth or differentiation (Seger and Krebs, 1995; Kyriakis and Avruch, 1996). The MAPKs include the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinase/stress activated protein kinases (JNK/SAPK) and the p38 MAPKs (p38s). These three families of MAPKs form three parallel signalling cascades activated by distinct and sometimes overlapping sets of stimuli. In general, ERKs are activated by mitogenic factors, while the JNKs and p38s are activated by stress-inducing agents or pro-inflammatory cytokines (Kyriakis and Avruch, 1996). Activation of p38 is regulated by small GTP-binding proteins (Chang *et al.*, 1995). Apoptotic signals can activate p38 via the ASK1 (MAPK kinase) (Ichijo *et al.*, 1997).

p38 Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38-mitogen-activated protein kinase (MAPK) pathways control the transcription and synthesis of TNF α stimulated by ionomycin (Hoffmeyer *et al.*, 1999). We have reported similar results (Mendis *et al.*, 1997), in which SEB activates all three of these MAPKs, with JNK the most activated in T cells. Inhibitors of these kinases block TNF α production, CD-69 expression and cell proliferation induced by SEB in human lymphoid cells. Both SE-induced p38 activation and TNF α release are inhibited by p38 inhibitors (Wadsworth *et al.*, 1999) and by cyclosporin A (Schafer *et al.*, 1999).

In addition to regulating IL-1 and TNF α synthesis by monocytes, p38 also controls several other cellular responses. For example, p38 activity is essential for the production of IL-10 and prostaglandin H synthase-2 (PGHS-2) by monocytes (Pouliot *et al.*, 1997; Foey *et al.*, 1998), and the production of PGHS-2, metalloproteinases and IL-6 by fibroblasts and endothelial cells (Ridley *et al.*, 1997; Beyaert *et al.*, 1996). P38 is constitutively active in mouse thymocytes, which suggests a role in T cell survival and/or differentiation (Sen *et al.*, 1996). IL-2 or IL-7 can induce an increase

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P38 is constitutively active in mouse thymocytes, which suggests a role in T cell survival and/or differentiation (Schafer *et al.*, 1999)

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in p38 activity in T cell lines (DeSilva *et al.*, 1997). Fas-mediated apoptosis of T and B cells is accompanied by p38 activation, but inhibition of p38 activity alone does not prevent cell death (Salmon *et al.*, 1997). Thus, it is becoming increasingly clear that p38 may participate in a variety of T cell responses.

The Jak/STAT Pathway The Jak/STAT pathway is utilised by conventional antigens, numerous cytokines and growth factors and by the SEs to signal from the cell membrane to the nucleus. Pathways that involve Jaks and STATs are important for haematopoietin receptor signalling. Many members of the cytokine receptor superfamily activate the Jak protein tyrosine kinase family, with resulting phosphorylation of the STAT transcriptional activator factors. Recent evidence for a preferential coupling of Jak3 to IL-2 receptor- γ (IL-2R γ) and Jak1 to IL-2R β supports the concept of that *trans*-activation of Jak1 and Jak3 is caused by IL-2-induced heterodimerisation of their receptor partners. SEA and SEB modulate IL-2 receptor (IL-2R) expression and signal transduction involving the Jak/STAT pathway (Nielsen *et al.*, 1995). The changes in the composition of IL-2Rs are accompanied by inhibition of IL-2-induced tyrosine phosphorylation of Jak3, STAT3 and STAT5, and IL-2-driven proliferation is inhibited significantly (Gerwien *et al.*, 1999). Mice exposed to SEA or SEB also show activation of the Jak/STAT pathway in their T cells (Gimeno *et al.*, 1996; Grundstrom *et al.*, 2000).

Other Signalling Molecules

Calcium Mobilisation A change in the level of calcium is an early event in a signal transduction pathway. SEA-induced, T cell-dependent calcium mobilisation in monocytes requires physical interactions between SEA-MHC class II, TCR/CD3 and CD4 molecules. SEB, SEA and TSST-1 mediate a T cell-dependent calcium increase in monocytes (Damaj *et al.*, 1992). The characteristics of the SEA-mediated calcium mobilisation in monocytes strongly support the hypothesis that this response is an integral part of the signal transducing machinery linked to MHC class II molecules. In cutaneous disorders, SEB may penetrate the epidermis and interact with HLA-DR-positive keratinocytes to up-regulate an adhesion molecule (ICAM-1) by calcium mobilisation, so contributing to the inflammatory process (Wakita *et al.*, 1995).

Role of Eicosanoids Arachidonic acid (AA) can be generated from hydrolysis of phospholipids by phospholipase A2 (PLA2). AA can be metabolised by the

cyclooxygenase pathway, resulting in production of highly potent bioactive lipids such as prostaglandins (PG) and thromboxanes, or it can be utilised by lipoxygenases, resulting in the production of leukotrienes (LT), lipoxins, and other highly potent molecules. A variety of AA metabolites possess the ability to modulate immune cell function. One of the most potent leukotrienes, LTB4, has long been known to be responsible for respiratory distress by causing broncho-constriction. Massively increased levels of LTB4 have been identified and well-characterised in asthmatics and patients with adult respiratory distress syndrome. Since one of the key features of SEB-induced shock is respiratory distress, it was recognised that leukotrienes are likely to be involved. They can be generated by many tissues, and lymphoid cells, well-established targets for the SEs, possess high levels of various lipoxygenases.

Numerous studies have shown very large increases in production of eicosanoids, especially leukotrienes, both *in vitro* and *in vivo* in response to SEB exposure (Jett *et al.*, 1990, 1992, 1994; Antonelli *et al.*, 1990; Boyle *et al.*, 1994; Henderson *et al.*, 1996). We have recently shown that there is a cross-talk between the AA pathway and the MAPK signalling pathway. Inhibitors of the 5-LO pathway block signalling of SEB through the stress-activated protein kinase pathway, suggesting a cross-talk between the two signalling pathways (Mendis *et al.*, 1998). This indicates that the AA pathway may act upstream of the MAPK pathway in SEB signal transduction.

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